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SUPPLEMENTARY INFORMATION

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The rise and fall of methanotrophy following a deepwater oil-well blowout

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Methods

Study Sites:

A total of 1128 water column samples were collected during ten research cruises to the Gulf of Mexico spanning March (the background sampling cruise) through December 2010, on board of the R/V Pelican: 1st Mar. 2010 – 7 Mar. 2010, 5th May 2010 – 15 May 2010 and 20th June 2010 – 26 June 2010; R/V F.G. Walton Smith: 25th May 2010 – 6 June 2010; R/V Nancy Foster: 30 June 2010 – 18 July 2010; R/V Oceanus and R/V Cape Hatteras: 21st Aug. 2010 – 16 Sept. 2010; R/V Arctic Sunrise: 20th Sept. 2010 – 28 Sept 2010; and R/V Atlantis: 8th Nov. 2010 – 3rd Dec. 2010 (See On-Line Supplementary Table). The pre-spill samples were collected from above a fracture zone at a natural hydrocarbon seep, Mississippi Canyon block 118, in March 2010, roughly a month before the Macondo Blowout began. It is important to note that the pre-spill data does not reflect the microbial abundance of an inactive site that lacks natural seepage inputs rather the data reflects a site impacted by low rates of natural seepage.

Sample collection:

A CTD-Niskin rosette system was used to obtain hydrographic profiles throughout the water column. Niskin bottles were triggered at specific depths and upon return to the surface, each bottle was individual samples were collected for dissolved gas

concentration determination, rates of aerobic methane oxidation, nutrient and metal concentration determination, and microbial molecular analysis (see below).

Methane concentration:

Samples for dissolved alkane quantification were collected as soon as the CTD rosette was secured on deck, as described previously by Joye et al.[31] with some modifications between cruises. Concentration of C_1 to C_5 alkanes were determined using headspace extraction (May through

or a modified sonication/vacuum extraction technique (August through December)[32], followed by gas chromatography. A sub-sample (0.25 to 1 mL) gas sample was injected into a gas chromatograph (model 8610C, SRI, California) equipped with a flame ionization detector[31]. A temperature ramp was employed to elute C_{3+} alkanes. Concentrations were calculated by comparison to a certified mixed alkane standard (C_1 to C_5, including both *n*- and *i*- C_4) (Scott Specialty Gases®).

Aerobic methane oxidation rates:

Water column aerobic methane oxidation rates were measured using a tritiated (^3H) CH_4 radiotracer technique[33,34] . Reactions were done in triplicates in gas-tight glass vials. A 100 µl aliquot of the C^3H_4 tracer solution was injected into each replicate yielding a tracer activity of 2 kBq. Killed controls were achieved by treating a sample with 3.7 % formaldehyde, to arrest microbial activity, prior to tracer addition. The samples were incubated at *in situ* temperature for 48 to 72 hours; linearity of activity was confirmed by time series. Reactions were terminated by adding 20% (vol:vol) of pure ethanol to each

vial. Labeled C^3H_4 was removed by purging the sample with hydrated air for at least 25 minutes. Scintillation cocktail (ScintiSafe Gel®) was then added to an aliquot of the sample and ^3H_2O produced was quantified using a Beckman 6500 liquid scintillation counter.

Methane stable carbon isotopic signature:

Water samples for methane stable carbon isotopic ($\delta^{13}C$) analyses (May and July) were collected into 125 mL serum vials. A headspace sub-sample from each vial was injected into a Finnegan Mat Delta V Isotope Ratio Mass Spectrometer coupled to a Hewlet Packard gas chromatograph with a poraplot capillary column. Samples were cryo-focused as described in Chanton and Liptay (2000)[35], and reported relative to Vienna Pee Dee Belemnite (VPDB) standard.

Nutrients/Biogeochemistry:

Fixation and analysis of water samples for quantifying dissolved ammonium (NH_4^+), inorganic carbon (DIC or HCO_3^-), nitrate plus nitrite (NO_x^-), and phosphate (PO_4^{3-}) followed previously described methods[36]. Samples for dissolved trace elements were collected using trace element cleaned, rosette-mounted, teflon-coated, external spring Niskin and Go-Flo bottles. Samples were cleanly syringe-filtered[37]. Metals were isolated from a small volume (3 mL) of sample using Mg-induced co-precipitation with added enriched ^{57}Fe and ^{65}Cu[38] and then analyzed using isotope-dilution by sector-field inductively coupled plasma-mass spectrometry.

DNA extraction:

Water samples for DNA extraction were filtered through a 0.22 µm Sterivex filter (Millipore) and stored frozen until extraction. The filter was extracted using the Ultra Clean Soil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions with few modifications. Two samples used for *pmoA* clone libraries (see section below) were extracted using different phenol:chloroform DNA extractions protcols; sample C40-6 was extracted following Vetriani *et al*. (1999)[39] while sample C59-9 was extracted according to Adams et al. (2013)[40]. Similar results from *pmoA* clone libraries were obtained, regardless of the extraction method used, see Fig. 3.

qPCR amplification:

Amplification of Type I relatives OPU1 and OPU3 was achieved with primer set putative_16S_OPU1F (5'-CAATGCCGCGTGTGTGAA-3') and putative_16S_OPU1R (5'-CCTCTCTTCCCCCGACTGAA-3') for OPU1, and primer set putative_OPU3F (5'-AGCACTTTCAATTGGGAGGAAA-3') and putative_OPU3R (5' GCCGGTGCTTCTTCTAAAGGT-3') for OPU3[41]. The unusual *pmoA* sequences were originally detected using monooxygenenase intergenic spacer analysis (hereafter, MISA)[42] and full length *pmoA* sequences were recovered subsequently following the strategy reported in Tavormina 2013[42]. The MISA assay was validated on cultured strains as reported in Tavormina et al.[42]. qPCR primers were developed from the full length sequence using Primer3 software http://frodo.wi.mit.edu/primer3/.

On linearized, CsCl-purified plasmid templates, these primer pairs demonstrate >95% amplification efficiency (as measured over 5 orders of magnitude template concentrations). Cross reactivity between target plasmid templates is <1%, however these primers may amplify related sequences in complex environmental samples. Quantitative PCR reactions were performed in a StepOne Real Time PCR System (Applied Biosystem), in a reaction consisting of 12.5µl of Power SYBR® Green PCR Master Mix, 1µl of template DNA (1-6 ng/µl), 0.625µl of BSA (10 ng/µl), 1.25µl of each primer (0.5µM final concentration), and water to a final volume of 25µl. qPCR programs consisted in a 95ºC initial denaturation for 10 min, followed by 40 cycles of 95ºC for 15 sec and a one step annealing/extension of 1 min at 57ºC for Type I methanotrophs 16S rRNA gene, as well as for OPU1 and OPU3, or 1 min at 60ºC in the case of Type II methanotrophs. The limit of detection for the qPCR assays was estimated to be 30 copies per reaction for Type I, 50 copies per reaction for Type II, 200 copies per reaction for OPU1, and 56 copies per reaction for OPU3. All the results were standardized to the volume (per mL) of seawater the DNA was extracted from.

Enumeration of *pmoA* phylotypes was performed in an ABI 7300 real time PCR system with the following conditions. The OPU3 *pmoA* phylotype was performed using primers, Taqman probe, and conditions as previously reported[42]. Primers and Taqman probe specific to the OPU1 *pmoA* phylotype (primers *pmoA*_OPU1qPCR_242f: 5'-TTACCCCGATCATGCTGGTT-3' and *pmoA*_OPU1qPCR_312r: 5'-GATTCTGAAGTGTTCCCAAACGA-3'. Probe: 5'-TTCCCAGCCGCTGTTCAGGCA-3') and primers specific to the novel *pmoA* phylotype (primers *pmoA*_spill_f: 5'-

AAACTATGAGTTTAACTGCTG-3' and *pmoA*_spill_r: 5'-TCAAAATTCCGCACAATCTTT-3') were developed using Primer Express v 2.0. OPU1 enumeration was performed using cycling conditions as reported previously for the OPU3 *pmoA* phylotype. To enumerate the novel phylotype, a SYBR assay (Power SYBR green, Applied Biosystems) was developed. 1µl each of template DNA, forward and reverse primer (0.5µM final concentration), and water were added to a final volume of 20µl. qPCR was performed with a 95ºC denaturation for 10 min, followed by 40 cycles of 95ºC (15") and an extension at 58ºC (1'). A final dissociation curve was included. For all functional gene assays, CsCl-purified linearized plasmid DNA was used as a standard. Primers displayed >95% amplification efficiency as measured over five orders magnitude and cross reactivity between target templates was below detection. Limit of detection for *pmoA* qPCR assays was 10 copies per reaction. The results were standardized to the mL of seawater.

Statistics:

A Wilcoxon Test was performed using Kaleidagraph (version 4.1) to determine the significance of the changes observed in qPCR results over the time.

Clone libraries, sequencing, and phylogenetic analysis:

pmoA gene fragments were amplified by polymerase chain reaction (PCR) from DNA obtained form eleven samples representing different time points and locations during our sampling campaign (Supplementary Table S2) using primer set wcpmoA189f and wcpmoA661r[43]. The PCR protocol consisted of an initial denaturation of 3 min at 94ºC,

followed by 28-40 cycles of 1 min at 94 ºC, 30s at 56 ºC, and 45s at 72ºC, ending with a final extension of 5 min at 72 ºC. The PCR master mix consisted of of 1-20 ng/µl DNA and either 1X GoTaq Flexi Reaction Buffer, 1.25 u of GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI), 2.5 mM $MgCl_2$, 0.25 mg/ml BSA, 100 µM dNTP mix, 1µM of each primer, and ddH_2O to a final volume of 25 µl; or OmniTaq DNA polymerase (0.5 µL), 1X Omni Taq reaction buffer, 100 µM dNTP mix, 1µM of each primer and ddH_2O to a final volume of 25 µl.

Amplified *pmoA* gene fragments were gel-purified using the QIAGEN Qiaquick gel extraction kit (Qiagen, Santa Clarita, CA, USA), and cloned into a pCR4-TOPO plasmid vector (Invitrogen, Inc., Carlsbad, CA, USA) at a 1:3 vector to insert ratio. The ligation products were transformed into *E. coli* One Shot Top10 competent cells (Invitrogen, Inc., Carlsbad, CA, USA). Ampicillin resistant clones were selected and grown up overnight at 37 ºC in freezing medium (LB + 100 µg/ml Amp + 10% glycerol). One mL of the clones was spun down and cell pellets were sent to Laragen, Inc (Culver City, CA) for Sanger sequencing. Sequences were also screened for closest relatives using the blastn application of the NCBI database. Sequences were aligned using Clustal X version 2.1 and manually adjusted using Seaview[44]. Operational taxonomic units (OTUs) were defined as sequences sharing more than 97% similarity and were calculated using MOTHUR[45].

Sequences were translated using the online tool EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/). The amino acid sequences were aligned with Clustal X version 2.1 and manually adjusted using Seaview[44]. Phylogenetic distances were calculated using the Observed Divergence matrix, and the neighbor joining method

was used to evaluate tree topologies. Phylo_win version 2.0 was utilized to plot tree topologies[44] whose robustness was tested by bootstrap analysis (1000 resamplings).

Supplementary Discussion

Evidence that Macondo Methane was Dispersed through the Water Column after June 2010:

The methane present in the water column at MC118 in June 2010 was distinct from the thermogenic methane that typifies the MC118 site (Bowles et al. 2011, Geochimica et Cosmochimica Acta). The mid-water methane concentration measured at MC118 in June 2010 had a $d^{13}C\text{-}CH_4$ of -53.8 ± 1.5 ‰ (n=5) and was as depleted as -55 ‰, which is significantly lighter than the average $d^{13}C\text{-}CH_4$ signature of methane at MC118 (-45‰) and outside the bounds of the range noted by the reviewer (-48 to -52 ‰). We have made nearly 100 measurements of methane concentration and isotopic composition in the water column above MC118 since 2010. Typically, methane concentrations are maximal near the seafloor and decrease with distance above the bottom. The concentrations measured in June 2010 were low except for the anomalous high concentration zone present in the mid-water; so we conclude that this methane was Macondo in origin.

Furthermore, other stations north of MC252, and east/northeast from MC118 also exhibited methane concentration >100 nM after July 2010. For example, we observed a methane concentration of 109 nM at 750 m at Cape Hatteras Station 68 (29.04, -88.12). This station is located 23 nm from MC252, which lead us to conclude that deepwater plume methane was dispersed upwards through the water column in a broad fashion.

185 Other examples of stations east/northeast of MC252 that exhibited methane
186 concentrations >100 nM are presented in a table on the following page.
187

Site/ Station	Date	Lat	Long	Distance to MC252 (nautical miles)	Heading from MC252 (degrees)	Depth (m)	Methane (nM)
CH065	11 Sept 2010	28.71	-87.93	24	94	309	138
CH066	12 Sept 2010	28.88	-87.93	26	70	719	132
CH066	12 Sept 2010	28.88	-87.93	26	70	567	157.
CH066	12 Sept 2010	28.88	-87.93	26	70	444	125
CH067	12 Sept 2010	29.04	-87.93	30	52	51	143
CH068	12 Sept 2010	29.04	-88.12	23	37	750	109
CH076	13 Sept 2010	29.24	-88.01	35	33	68	174
CH077	13 Sept 2010	29.23	-87.82	42	44	212	125
CH077	13 Sept 2010	29.23	-87.82	42	44	186	141

Data Availability:

All of the data presented in this paper are available electronically through the Gulf of Mexico Research Initiative's Data Warehouse (https://data.gulfresearchinitiative.org/).

Supplementary References

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Captions for Supplementary Figures

Supplementary Fig. 1. **Map showing the study sites over space and time**. The sampling sites as a function of time since the Deepwater Horizon exploded are shown (colour code denotes time). The star denotes the location of the Macondo wellhead.

Supplementary Fig. 2. **Average methane concentration over 50-day periods**. Maximum methane concentrations over time, binned in 200m depth and 50 day intervals (colour coding is the same as Fig. 1) illustrating dispersion of the deep-water plume over time.

Supplementary Fig. 3. **Dissolved alkane concentrations in the water column during May and June 2010**. Concentration of C_2-C_5 alkanes (time colouring extends to day 50 only as concentrations were below detection after that time; colour coding same as Figs. 1.

Supplementary Fig. 4. **Dissolved nutrient concentrations in the water column over the 10-month time-course.** Concentration of ammonium, nitrate+nitrite, and phosphate over depth (all concentrations are in μM) for the various study sites are shown.

Supplementary Table 1.

Date	Research Vessel	Site / Station	Lat	Long	ID	Depth (m)	Extraction Method	Remarks
12-May-10	Pelican	Site_39	28.70	-88.39	WC45	1265	MoBio Kit	Combined DNA
14-May-10		Site_33A	28.72	-88.42	WC75	1220	MoBio Kit	
14-May-10	Pelican	Site_34A	28.71	-88.39	WC65	1020	MoBio Kit	Individual sample
26-May-10	Walton Smith	WS2	28.73	-88.41	C4-9	1120	MoBio Kit	Combined DNA
4-Jun-10		WS6	28.74	-88.38	C73-3	1180	MoBio Kit	
30-May-10	Walton Smith	WS36	28.71	-88.41	C40-6	1220	Vetriani *et at.*, 1999	Individual sample
1-Jun-10	Walton Smith	WS53	28.73	-88.38	C59-3	1170	Adams *et al.*, 2013	Individual sample
9-Sep-10	Oceanus	022.01	29.00	-88.80	179	162	MoBio Kit	Individual sample
30-Aug-10	Oceanus	014.02	27.37	-90.56	101	1222	MoBio Kit	Individual sample
30-Aug-10	Oceanus	014.02	27.37	-90.56	102	1000	MoBio Kit	Individual sample
30-Aug-10	Oceanus	014.02	27.37	-90.56	103	700	MoBio Kit	Individual sample
13-Sep-10	Oceanus	027.11	28.55	-88.32	242	1289	MoBio Kit	Individual sample